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Impact of hyperpigmentation on superoxide flux and melanoma cell metabolism at mitochondrial Complex II

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Short title: Hyperpigmentation, superoxide and mitochondrial Complex II

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Abbreviations:

UVR; ultraviolet radiation, ROS; reactive oxygen species, IM; isolated mitochondria, O_2^- ; superoxide, TTFA; thenoyltrifluoroacetone, 3-NP; 3-nitropropionic acid, FCS; foetal calf serum, DTSSP; dithiobis(sulfosuccinimidyl propionate, SDS-PAGE; Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, HRP; horseradish peroxidase, ECL; electrochemiluminescent.

Abstract

Melanogenesis is a highly conserved process of cytophotoprotection from ultraviolet radiation (UVR) present in many species. While both mitochondrial function and UVR insults are both well documented promoters of increased cellular stress, their individual molecular relationships with skin pigmentation have not been clearly resolved. This study provides evidence for a direct relationship between cellular melanin content, superoxide flux and mitochondrial function at Complex II. Direct and significant correlation between increased pigmentation and Complex II turnover was observed in genetically different melanoma cell lines of varied basal pigmentation states ($p<0.01$). The same trend was also observed when comparing genetically identical cell cultures with increasing levels of induced pigmentation ($p<0.005$). The observation of increased steady-state levels of the catalytic Complex II subunit SDHA alongside hyperpigmentation suggested co-regulation of activity and pigment production ($p<0.01$).

The study also presents novel evidence for a relationship between hyperpigmentation and increased O_2^- generating capacity at Complex II. By amperometrically monitoring superoxide flux from differently pigmented FM55 melanocytes and their isolated mitochondria, a dynamic and responsive relationship between pigmentation, Complex II function and intracellular superoxide generation was observed ($p<0.005$). The data supports hyperpigmentation as a protective antioxidant mechanism in response to Complex II-mediated ROS generation.

Keywords: Bioenergetics, melanin, succinate dehydrogenase, skin, cytoprotection.

Introduction

The production of melanin by cutaneous melanocytes as a cytoprotective mechanism is an energy intensive process that puts significant demand upon cellular ATP reserves. While sunlight exposure is a physiological necessity for endogenous vitamin D production, overexposure has been unequivocally related to sunburn, premature ageing and skin cancer incidence. We and others have previously shown that UVR irradiation causes increased mitochondrial DNA (mtDNA) mutations and reactive oxygen species (ROS) generation in skin, which has led to pioneering the use of mtDNA damage as a biomarker of UVR exposure in human skin (1-4). Such damage can compromise mitochondrial ATP generation efficiency and further exacerbate intracellular oxidative stress as electrons leak from sites at, or within, the respiratory complexes (5, 6). Although links between cellular oxidative stress and pigmentation state have been reported, a molecular relationship remains elusive (7-9). As such, we ascertained if pigmentation responses directly impacted upon mitochondrial electron transport chain (mETC) complex activities and their capacity for O_2^- production.

A previous investigation within our group reported real-time monitoring of mitochondrial superoxide (O_2^-) flux by recording the current generated by direct interaction of O_2^- with the surface of a cytochrome *c* functionalised gold electrode (10). Addition of Complex I and III inhibitors (rotenone and antimycin A respectively) to an isolated mitochondrial suspension brought about an increase in current that was quenched by the addition of superoxide dismutase. The current study initially extended the previously reported work by investigating differences in inducible O_2^- flux in differently pigmented cell lines. A relationship between inducible O_2^- flux at Complex II and basal cellular melanin content was identified. This relationship was also observed in genetically identical melanoma cells that were treated to increase cellular melanin content. Western blotting provided data postulating that Complex II activity and O_2^- flux may be regulated by the induction of SDHA, the catalytic subunit of Complex II.

Materials and methods

Reagents

Cell culture reagents were purchased from Lonza (Cambridge, UK). The thiol cross-linker 3-3'-dithiobis(sulphosuccinimidylpropionate) (DTSSP) was supplied by Pierce (Chester, UK). Primary and secondary antibodies were supplied by AbCam (Cambridge, UK). All other reagents were purchased from Sigma Aldrich (Poole, UK).

Cell culture and hyperpigmentation treatment

The three human melanoma cell lines used (minimally pigmented CHL1, moderately pigmented FM55 and highly pigmented FM94) were routinely cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 U.ml⁻¹ penicillin G and 100 µg.ml⁻¹ streptomycin. Cells were maintained in a humidified environment containing 5% (v/v) CO₂ at 37°C. Cells were grown to 80% confluence and passaged 8 times, with mitochondrial isolation at the third and sixth passage. Hyperpigmentation was induced using 400µM L-tyrosine and 10 mM NH₄Cl in their culture medium for up to two days or 200µM L-tyrosine and 5 mM NH₄Cl concentrations for upto 4 days with cell sampling on a daily basis from treatment initiation. Melanin concentration was determined as necessary using a previously defined method (9) with prior protein concentration analysis by Bradford assay (Biorad, Hertfordshire, UK) as per the manufacturer's instructions. Cell samples were standardised to 1mg.ml⁻¹ protein prior to melanin analysis, and absorbance at 475nm was carried out using a Nanodrop microspectrophotometer (Thermo Fisher, Loughborough, UK) with associated software.

Mitochondrial fraction preparation

Mitochondrial fractions were prepared as previously reported (10). Prior to storage, the protein concentration of the sample was determined using a Bradford assay (Bio-Rad, UK) as per the manufacturer's instructions. Mitochondrial fractions were kept in sucrose-rich buffer throughout all electrochemical experiments.

Determination of mitochondrial enzyme activities

Determination of citrate synthase, Complex II and Complex IV activity in both isolated mitochondria and whole cell lysate were determined with identical spectroscopic methodology as described by Kirby *et al* (11) and based upon those established by Birch-Machin *et al* (12).

Preparation of O₂⁻ specific electrode

The electrode was functionalised and calibrated as previously described (10, 13). For all experiments, the electrode was poised at an operating potential of +100 mV against a steel counter electrode using a Ag/AgCl

reference electrode (Harvard Apparatus, Kent, UK). An Autolab PGSTAT101 single channel potentiostat was used throughout all experimentation and data was recorded using the associated Nova datalogging software.

Amperometric monitoring of O_2^- flux from isolated mitochondria and whole cell samples.

Aliquots of isolated mitochondria were adjusted to 1000 U.ml⁻¹ citrate synthase activity per ml using sucrose-rich mitoprep buffer to standardise mitochondrial content across all samples tested. 100µl aliquots of mitochondrial suspension were presented to the functionalised O_2^- selective electrode in a custom made conical dish. Prior to whole cell investigations, cells were seeded into 30mm tissue culture dishes containing 3 x 16 mm cover slips. Dishes were treated in triplicate with hyperpigmentation medium as required. 3 control dishes did not receive any treatment. Individual cover slips were removed and presented to the electrode in a conical testing well with 1 ml medium. The electrode was poised at +100 mV working potential vs. a steel counter using a Ag/AgCl reference electrode and any changes in current were recorded using a PGSTAT101 single channel potentiostat (Metrohm UK, Cheshire, UK) and associated NOVA datalogging software. The electrode was submerged in the presented sample medium and data logging was commenced. Upon baseline current stabilisation, 100µM inhibitor (either rotenone, TTFA, 3-NP or antimycin A) was added to the conical well. When challenging whole cells, 300µM DDM was added prior to inhibitor addition to permeabilise the cells membranes. All experiments were repeated in triplicate with current changes recorded for 5 minutes following inhibitor addition. Individual traces were corrected using a baseline regression to account for electrode drift. Peak current was consistently observed between 10 and 30 seconds following inhibitor addition. This peak response was used as a comparative marker for O_2^- flux across all challenged samples.

Western Blotting for Complex II subunits

Total protein was extracted into 0.1% SDS in PBS with 10s sonication. Insoluble debris was removed by centrifugation (8000xg for 3 min). Total protein was determined using a detergent compatible Bradford Assay Kit (Bio-Rad, Hertfordshire, UK) as per the manufacturer's instructions. 10µg protein per sample was loaded into a 4-20% SDS-PAGE gel (Life Technologies, Paisley, UK) and was separated under the manufacturer's suggested conditions. Proteins were electrotransferred to a nitrocellulose membrane using a Transblot Turbo unit (BioRad, Hertfordshire, UK) at the manufacturer's standard settings. The transferred membrane was probed with primary (SDHA (0.1µg.ml⁻¹), SDHB (0.2µg.ml⁻¹) or β-actin (1µg.ml⁻¹)) and associated secondary antibodies (HRP-conjugated rabbit anti-mouse, 0.1µg.ml⁻¹) in conjunction with Dura ECL substrate (Thermo Fisher, Loughborough, UK) Densitometric measurements were made from developed Amersham Hyperfilm (Thermo Fisher, Loughborough, UK) using Alpha Innovatech camera unit and associated Fluorochem software.

Results

Amperometric monitoring of O_2^- flux induced by mETC inhibitors in mitochondrial fractions isolated from differently pigmented cell lines.

The addition of respiratory inhibitors to a suspension of isolated mitochondria in sucrose rich buffer has previously been reported to cause electron leakage and subsequent O_2^- formation (10). O_2^- flux from mitochondria isolated from the differently pigmented melanoma cell lines CHL1 (minimally pigmented), FM55 (moderately pigmented) and FM94 (highly pigmented) was monitored using an established amperometric technique (10). Prior to inhibitor addition, mitochondrial fractions were normalised to 1000 U citrate synthase activity per ml to ensure mitochondrial content standardisation (11, 12). As demonstrated by Figure 1, rotenone and antimycin A generated the largest currents following inhibitor addition, however there were no significant differences in response to either inhibitor between the 3 parent cell lines ($p > 0.05$). In contrast, Complex II inhibition with TTFA and 3-NP elicited significantly different mean mitochondrial responses across the 3 parent cell lines ($p < 0.05$). In particular, a 3.8 fold greater average O_2^- current was generated by the highly pigmented FM94 cell line mitochondria than those from the minimally pigmented CHL1 line in response to TTFA. Similarly, the comparable experiment using 3-NP inhibition of Complex II generated 2.8 fold higher average O_2^- current in FM94 mitochondria versus CHL1 mitochondria.

Effects of basal cellular melanin content on Complex II mediated O_2^- flux following enzyme activity inhibition.

The observation of differing mitochondria derived O_2^- fluxes from inhibited Complex II between the differently pigmented cell lines (Figure 1) was further investigated. Data clarity and efficacy were improved by employing a recently described Complex II specific normalisation procedure (14) to express the specific impact of Complex II regarding O_2^- flux as a function of the sample's innate Complex II activity. For each mitochondrial fraction, Complex II activity was determined and used to normalise corresponding Complex II mediated O_2^- flux. This normalisation removed confounding factors introduced by innate enzyme activity variation, permitting direct comparison of O_2^- generating capacity across the cell lines. The resulting O_2^- generated current values were plotted against basal melanin concentration values determined for each cell line thereby enabling a quantitative comparative analysis. The data in Figure 2 clearly shows significant positive correlation between increasing melanin content and O_2^- generated current following the Complex II inhibition with TTFA and 3-NP.

Complex II activity increases with FM55 and CHL1 cell hyperpigmentation.

The observations in Figure 2 prompted further investigation of the relationship between Complex II activity and melanin content of the cultured cells from which the mitochondria were isolated. To avoid confounding subtle observations with cell type and genetic background dependent metabolic variations, Complex II activity was monitored following gradual hyperpigmentation of the moderately pigmented melanoma cell line FM55. This

was achieved by culturing cells in the presence of L-tyrosine and NH_4Cl as previously described (15). The minimally pigmented melanoma cell line CHL1 was investigated simultaneously using the same hyperpigmentation protocol for comparison. The resulting increased melanin content was determined accordingly. Complex II activity was determined in permeabilised whole cells and was normalised to citrate synthase activity for the reasons previously described (12). Complex IV activity was also determined in FM55 samples, however no relationship between melanin content and activity was observed (Pearson $r = -0.1355$, $p > 0.5$; data not shown).

The data presented in Figure 3 demonstrates the observed 1.6 fold increase in Complex II activity was associated with a maximum 5.4 fold increase in cellular melanin concentration following hyperpigmentation of the FM55 cells. Investigation of the CHL1 cells alongside the FM55 line corroborated the observed association of pigmentation and Complex II activity, with a 6.5 fold increase in Complex II activity associated with maximal hyperpigmentation. Unsurprisingly, lower absolute pigment concentrations were recorded in the basally minimally pigmented amelanotic CHL1 cells (4.2 fold maximum increase) compared to the FM55 cells as a consequence of the hyperpigmentation protocol. This fold change in pigmentation however is comparable to maximally hyperpigmented FM55 cells. Significant correlation between cellular melanin content and Complex II to citrate synthase activity ratio was observed for both cell lines over all treatment conditions tested.

Steady-state levels of SDHA subunit increase with FM55 cell hyperpigmentation.

A functional rationale for the increased Complex II activity alongside hyperpigmentation presented in Figure 3 was investigated using the FM55 cell line. Cell samples were hyperpigmented using the previously described process and analysed by western blotting to elucidate steady-state levels of two major Complex II subunits, namely SDHA (catalytic flavoprotein subunit) and SHDB (iron-sulphur protein subunit), and a generalised cell housekeeper protein, β -actin. Pearson product-moment correlation analysis revealed a significant relationship between cellular melanin content and steady-state levels of SDHA (Pearson $r = 0.9725$, $p < 0.01$) (Figure 4A). In contrast, there was no relationship observed between steady-state SDHB levels and cellular melanin content. Hyperpigmentation treatment to induce a 5.5 fold increase in cellular melanin content also induced a 4.4 fold increase in steady-state SDHA level compared to the non-hyperpigmented population (Figure 4A&B). This suggested an activity modulation role for SDHA which houses the catalytic centre for the enzyme and thus would impact upon the observed Complex II activity changes reported in Figure 3.

Dynamic O_2^- generation of progressively hyperpigmented FM55 cells following Complex II inhibition.

Data presented in Figure 3 clearly demonstrated an increase in Complex II activity concurrent with FM55 cell hyperpigmentation. This appeared to be associated with increased catalytic SDHA subunit steady-state levels (Figure 4). Interestingly, a recent investigation has shown that Complex II is capable of ROS generation rates

comparable to other sites at Complexes I and III and is a more important ROS contributor than previously thought (14, 16). This scenario was also supported by preliminary data reported by our group (17). Increased Complex II activity (as reported in Figure 3) is therefore likely to promote increased ROS generation, which can be exacerbated by inhibition of Complex II activity with addition of 3-NP and TTFA to facilitate measurement. The whole cell scenario of increased O_2^- associated with increased Complex II activity and hyperpigmentation is not straightforward. Our group has previously reported that increased pigmentation of the FM55 cell line protected against mitochondrial superoxide generation and mitochondrial DNA damage (9). Dynamic control of intracellular ROS production has been suggested, particularly surrounding the balance between increased flux as a result of increased Complex II activity and the protective effect of melanin. This dynamic was investigated by amperometric monitoring of TTFA and 3-NP induced O_2^- flux from increasingly pigmented FM55 cells. O_2^- generated current from hyperpigmented cells is presented in Figure 5 as a percentage of current recorded from basally pigmented FM55 control cells treated with O_2^- flux stimulating inhibitors. An immediate 2.5 fold increase in inducible O_2^- flux was observed from cell samples containing $89\mu\text{g.mg}^{-1}$ protein melanin. This melanin content was representative of cells that received 24hr hyperpigmentation treatment with $200\mu\text{M}$ L-tyrosine and 5mM NH_4Cl . Subsequently, cell populations displaying progressively higher melanin contents reproducibly generated less O_2^- in response to TTFA and 3-NP. Furthermore, cell samples with the highest melanin concentration of $406\mu\text{g.mg}^{-1}$ protein generated O_2^- flux comparable to original basal levels. The results presented in Figure 5 suggested that following an expected initial increase in inducible flux as a result of hyperpigmentation, further increases in melanin content serve to progressively decrease the observed inducible O_2^- flux to baseline levels. This is probably due to the protective effect of melanin becoming more prominent during hyperpigmentation, which in turn increases the antioxidant capacity of the cells and overcomes the increased mitochondrial O_2^- flux.

Discussion

This study aimed to investigate the relationship between hyperpigmentation, mitochondrial metabolism and O_2^- flux based on recent literature from our group that linked higher basal pigmentation to a reduction in mtDNA damage and ROS in UVR-irradiated cells (9). Amperometric techniques established within the group were used alongside kinetic, spectroscopic and western blotting techniques to provide corroborating evidence for this dynamic relationship within human melanoma cell models.

Our group's previous amperometric study established a method using a cytochrome *c* functionalised gold electrode for the real-time monitoring of O_2^- flux in mitochondria isolated from the human melanoma FM55 cell line. While the previously published study (10) presented proof of concept data for monitoring O_2^- in real-time from isolated mitochondria, it utilised mitochondrial fractions isolated from a single melanoma cell line and only 2 sites of O_2^- flux (Complex I and Complex III). The current study corroborated and greatly extended this work by comparing the O_2^- flux following inhibition of 4 different mETC sites across 3 differently pigmented melanoma cell lines. The study presents the first reported amperometric study of O_2^- from hyperpigmented but genetically identical cell populations (both FM55 and CHL1 melanoma cell lines). To the best of our knowledge, this work also represents the first time amperometric O_2^- sensing techniques have been used to monitor directly induced mitochondrial O_2^- flux from permeabilised whole cells as opposed to isolated mitochondrial fractions.

No significant differences were identified between parent cell line and inducible O_2^- flux when investigating Complexes I and III. Significant differences were however observed between differently pigmented cell lines following Complex II inhibition with TTFA and 3-NP (Figure 1). Furthermore, it was found that O_2^- flux elicited by Complex II inhibition corresponded to cell basal pigmentation state when normalised to Complex activity (

Figure 2). When investigated in the context of hyperpigmented, genetically identical whole cells rather than isolated mitochondria, significant positive correlation was observed between increased cellular melanin content and increased Complex II activity (

Figure 3: The activity of mitochondrial Complex II was ascertained over the course of hyperpigmentation treatment using spectrophotometric kinetic analysis as previously described (12). Hyperpigmentation of the FM55 cell line lead to a significant graded 1.64 fold increase in Complex II activity (6.5 fold increase in CHL1), with a maximum 5.4 fold increase in cellular melanin concentration (4.2 fold in CHL1 cells). A significant positive relationship between pigment concentration and Complex II activity was suggested by Pearson product-moment correlation analysis of both FM55 cells (Pearson $r=0.9330$, $p<0.005$) and CHL1 cells (Pearson $r=0.9418$, $p<0.005$).

Hyperpigmentation of both the FM55 and CHL1 melanoma cell lines with an established protocol (9, 15) resulted in a progressive increase in melanin content and a concurrent increase in Complex II activity (

Figure 3). The treatment is proposed to increase melanin content by increasing melanosomal pH via NH_4Cl mediated proton pump inhibition (18) and providing additional substrate for melanin production in the form of L-tyrosine. The hyperpigmentation protocol allowed metabolic analysis of genetically identical cells with different pigmentation states. The observed effects were corroborated by hyperpigmentation of the ordinarily minimally pigmented melanoma cell line CHL1 using an identical regimen (Figure 3). In both cell lines, the observed increases in Complex II activity correlated significantly with cellular melanin content. These findings were consonant with recent studies postulating the optimal pH for melanogenesis as nearer neutral rather than the acidic (~pH4) innate melanosomal environment (15, 18, 19). The observation of increased pigmentation of a reportedly minimally pigmented cell line with NH_4Cl treatment is agreement with postulated links between intramelanosomal pH and tyrosinase activity (18).

Western blotting for SDHA and SDHB subunits of Complex II in FM55 cells revealed elevated steady-state levels of the catalytic SDHA subunit only (

Figure 34); however this suggestion of active upregulation was only significant at the extremes of pigmentation. The observed increase in SDHA mirrored the trend in increased Complex II activity and increased melanin content. This finding was indicative of a potential regulatory pathway responsible, at least in part, for Complex II activity modulation. SDHAF2, the catalytic protein responsible for the flavination of the SDHA subunit (also termed SDH5) (20, 21), presents a rapid route to activity augmentation should the assembled complex be present in a latent state in the inner mitochondrial membrane. Investigation of this protein is still a relatively early stage.

The relationship between Complex II-mediated O_2^- flux, Complex II activity and hyperpigmentation is more convoluted than initially hypothesised. Maximum inducible O_2^- flux from permeabilised hyperpigmented FM55 cells initially increased profoundly to an average of 2.4 fold higher than control levels with a 1.6 fold increase in melanin content. This increase was induced using the lowest concentration hyperpigmentation treatment for the shortest incubation time (200 μ M L-tyrosine and 5 mM NH_4Cl for 24 hours). Relative inducible flux was incrementally reduced with further hyperpigmentation, reaching basal levels in the most highly pigmented samples (~406 μ g.mg⁻¹ protein). This data suggested a dynamic quasi-equilibrium between pigmentation state and Complex II turnover. Whilst innately darker cells appear to have a lower oxidative state, the observation could in fact be the result of higher antioxidant capacity masking an elevated leakage of electrons from more metabolically active mitochondria. This may in part be driven by increased Complex II activity. TTFA and 3-NP were used to block Complex II and enhance ROS production at and upstream of Complex II. TTFA is an incomplete inhibitor binding at the (distal) site of ubiquinone cycling whereas 3-NP is a competitive inhibitor at the succinate binding (proximal) site (22-24). Both inhibitors were capable of inducing increase O_2^- flux, and as such were used in combination within this study to give a more comprehensive overview of site specific O_2^- flux than the use of either inhibitor alone would allow. The modulation of O_2^- flux from hyperpigmented cells may further support our hypothesis that increased pigment production may be important for dissipating increased electron leakage (and as such reducing the proximal formation of O_2^-) from more metabolically active mitochondria during a tanning response. Interestingly, a recent mouse study stated that pigment production following UVA irradiation exacerbated melanoma induction. Concurrent with existing literature proposing two independent pathways to melanomagenesis corresponding to induction by UVA and UVB irradiation respectively, the study found that albino mice on the same genetic background developed fewer tumours than their pigmented counterparts following UVA irradiation. There were no significant differences between the mouse model tumourigenesis following UVB exposure (25). In line with current understanding that aberrant melanin synthesis is indeed associated with an increase in oxidative stress (26), the authors proposed that the increase in melanomagenesis observed in black mice was a result of dysregulated melanin synthesis induced by UVA exposure. In extension to this observation, our current study suggests that there may be a mitochondrial

basis for the acute increase in oxidative stress observed, particularly though the induction of Complex II activity.

Clearly, the relationship between metabolism, melanin production and stress responses is highly dynamic and incompletely defined. Future studies to provide greater insights to the temporal and mechanistic processes involved may demonstrate a possible rationale for the Warburgian environments and oncogenesis associated with Complex II aberration (20, 27), however it remains too early to fully elucidate the full impact of this subtle but highly important relationship.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure Legends

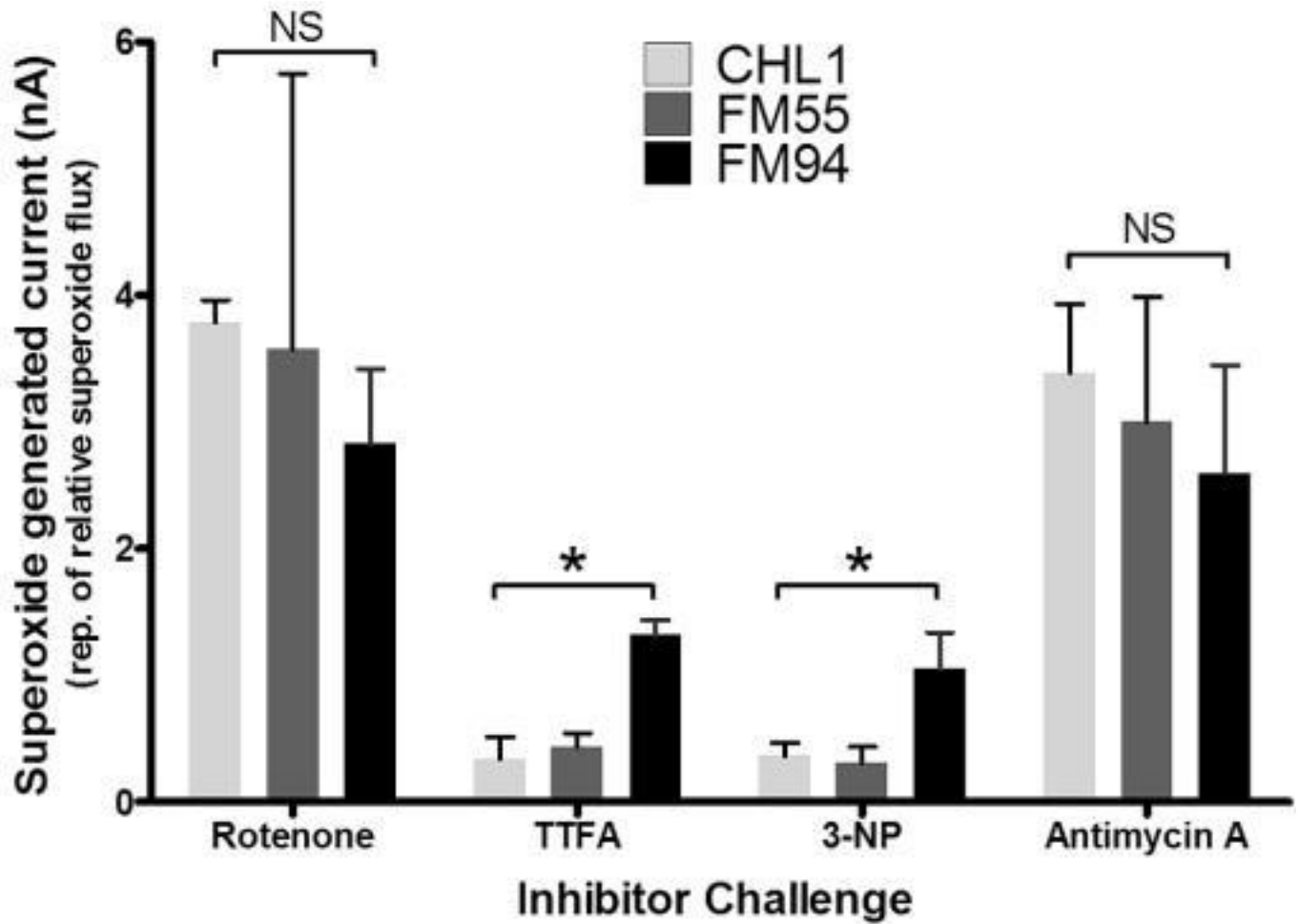


Figure 1: Isolated mitochondrial fractions (normalised to 100U citrate synthase activity) from 3 melanoma cell lines of different pigmentation state were challenged with inhibitors of Complexes I-III of the mETC. The maximum flux of O_2^- elicited by each inhibitor was recorded as an increase in current from a stable baseline using the previously reported amperometric technique (10). No relationship was observed between O_2^- flux and Complex I or III inhibition with rotenone and antimycin A respectively ($p > 0.05$, $n = 3$) however significantly different mean fluxes were observed following Complex II inhibition by both TTFA and 3-NP ($p < 0.05$, $n = 3$; denoted by *). 1-way ANOVA testing was used throughout. Data presented \pm SEM.

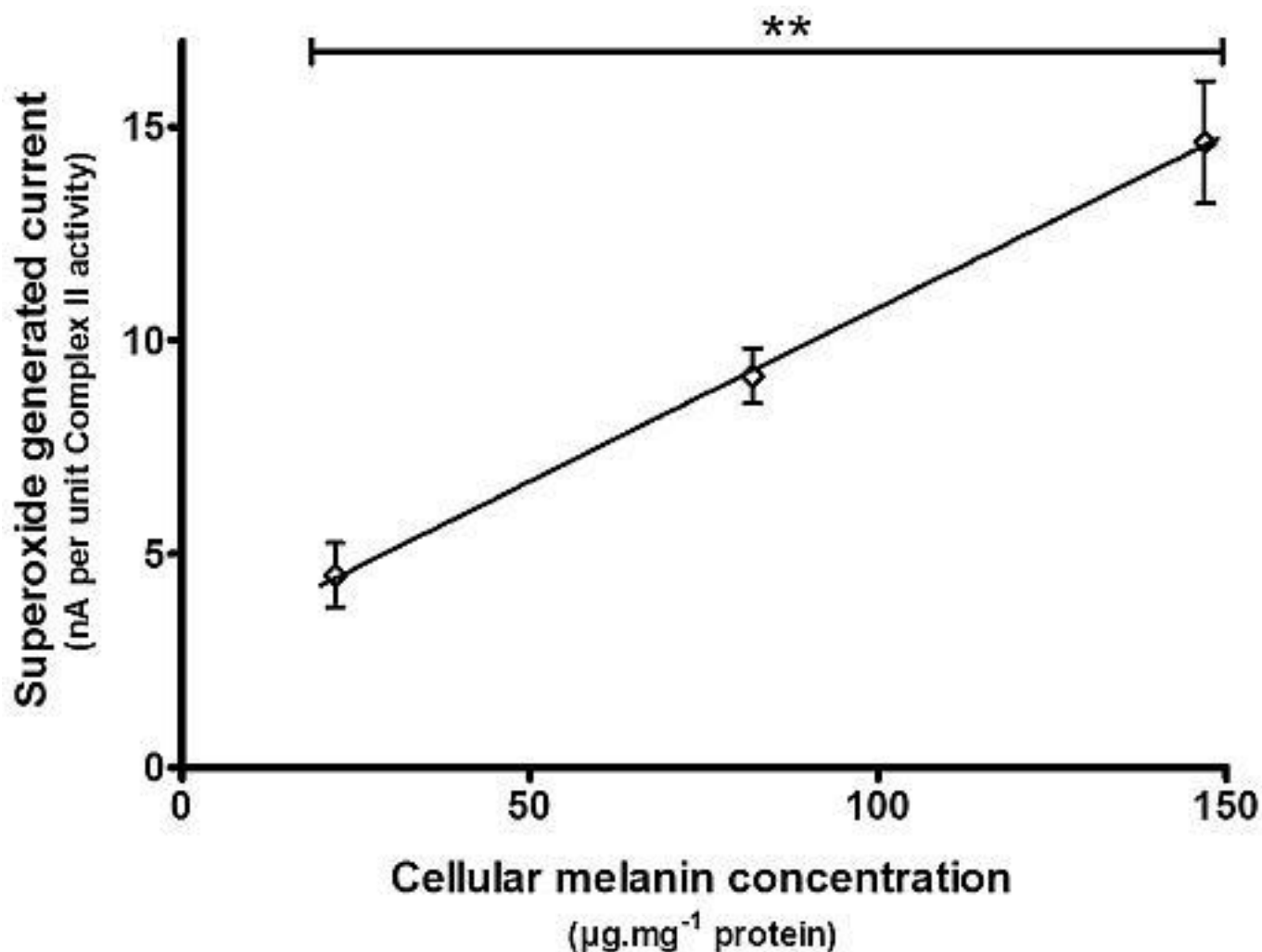


Figure 2: The O_2^- flux from isolated mitochondria from differently pigmented cell lines was assessed using a previously reported amperometric technique (10) and expressed as a function of the innate Complex II activity in the mitochondrial samples. When normalised to Complex II activity, a significant linear trend between parent cell pigmentation and Complex II elicited O_2^- flux was observed. ** denotes significant correlation ($p > 0.01$) following Pearson product-moment correlation analysis (Pearson $r = 0.9997$. Data presented \pm SEM, $n = 3$).

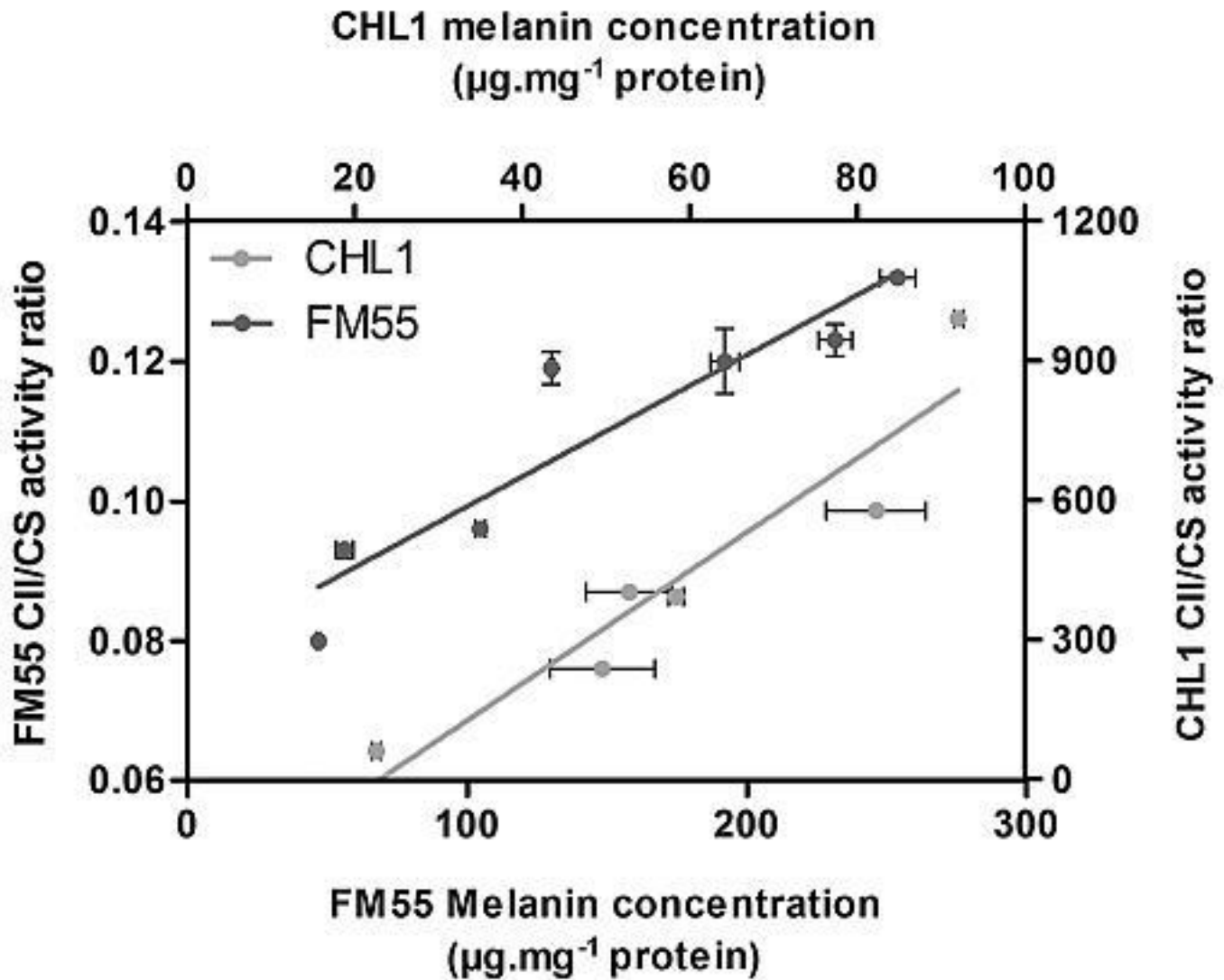


Figure 3: The activity of mitochondrial Complex II was ascertained over the course of hyperpigmentation treatment using spectrophotometric kinetic analysis as previously described (12). Hyperpigmentation of the FM55 cell line lead to a significant graded 1.64 fold increase in Complex II activity (6.5 fold increase in CHL1), with a maximum 5.4 fold increase in cellular melanin concentration (4.2 fold in CHL1 cells). A significant positive relationship between pigment concentration and Complex II activity was suggested by Pearson product-moment correlation analysis of both FM55 cells (Pearson $r=0.9330$, $p<0.005$) and CHL1 cells (Pearson $r=0.9418$, $p<0.005$).

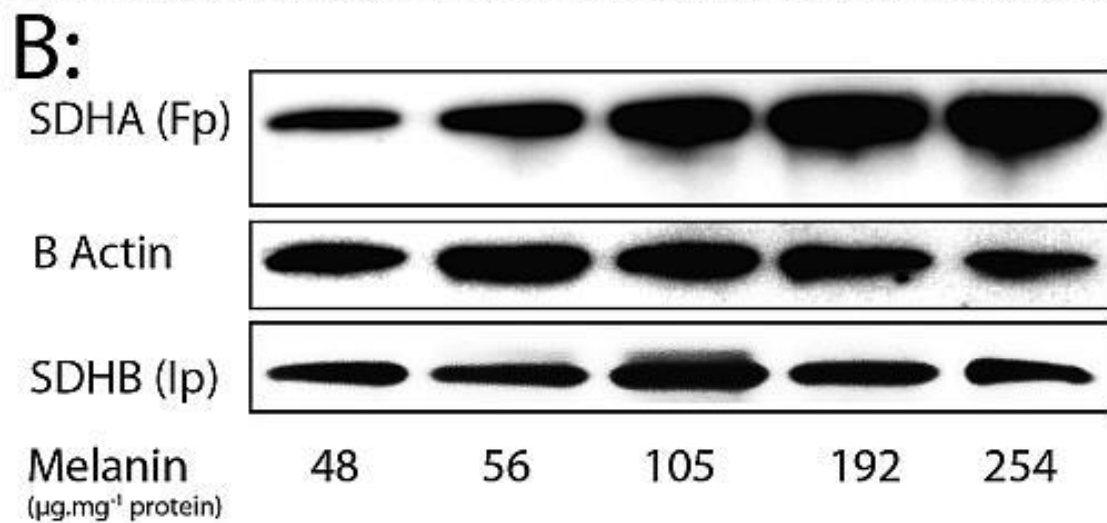
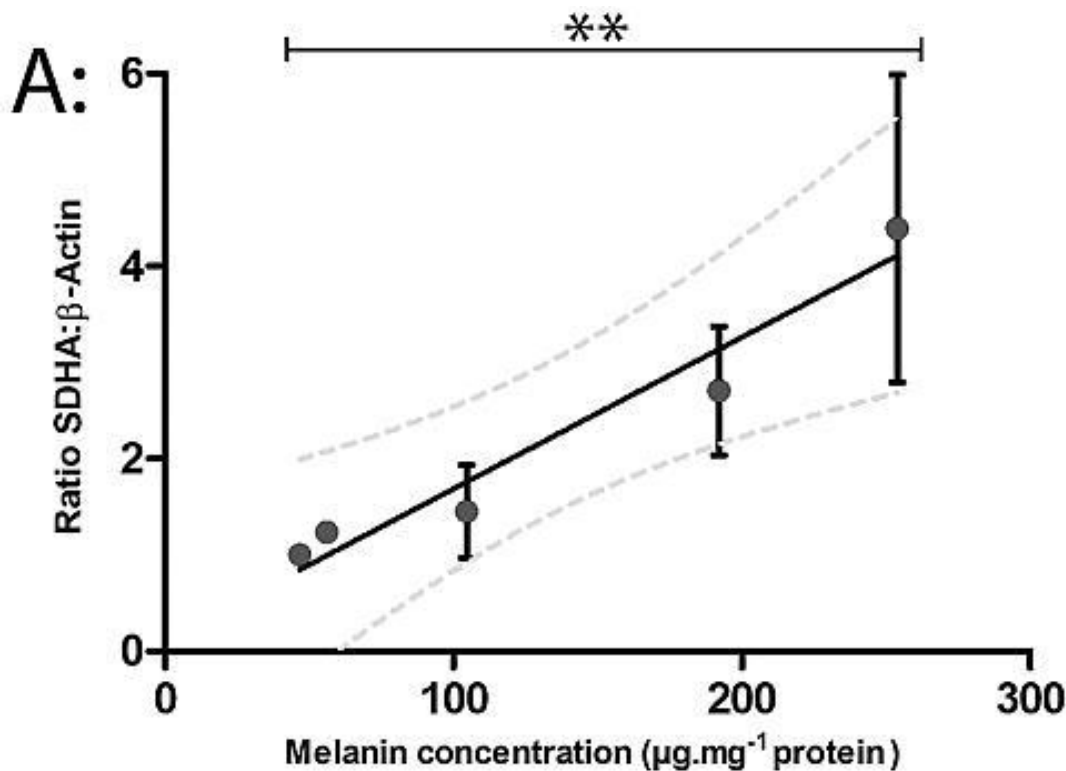


Figure 4: Analysis of the steady-state levels of Complex II subunit SDHA in hyperpigmented FM55 cells. A: Over the observed range of pigmentation (melanin concentration), linear regression analysis supported a positive trend in steady-state protein levels that deviated significantly from zero ($p < 0.005$), whilst correlation analysis revealed a significant relationship between cellular pigmentation state and SDHA steady-state level (Pearson $r = 0.9725$, $p < 0.01$). No such relationships were observed for SDHB (data not shown). B: A representative western blot demonstrating the increase in steady-state levels of SDHA compared to housekeeper β -actin and SDHB. The melanin concentration of the cell lysate used for the blot was determined and was displayed under the corresponding sample lane. Images representing each of the 3 proteins analysed were taken from the same blot.

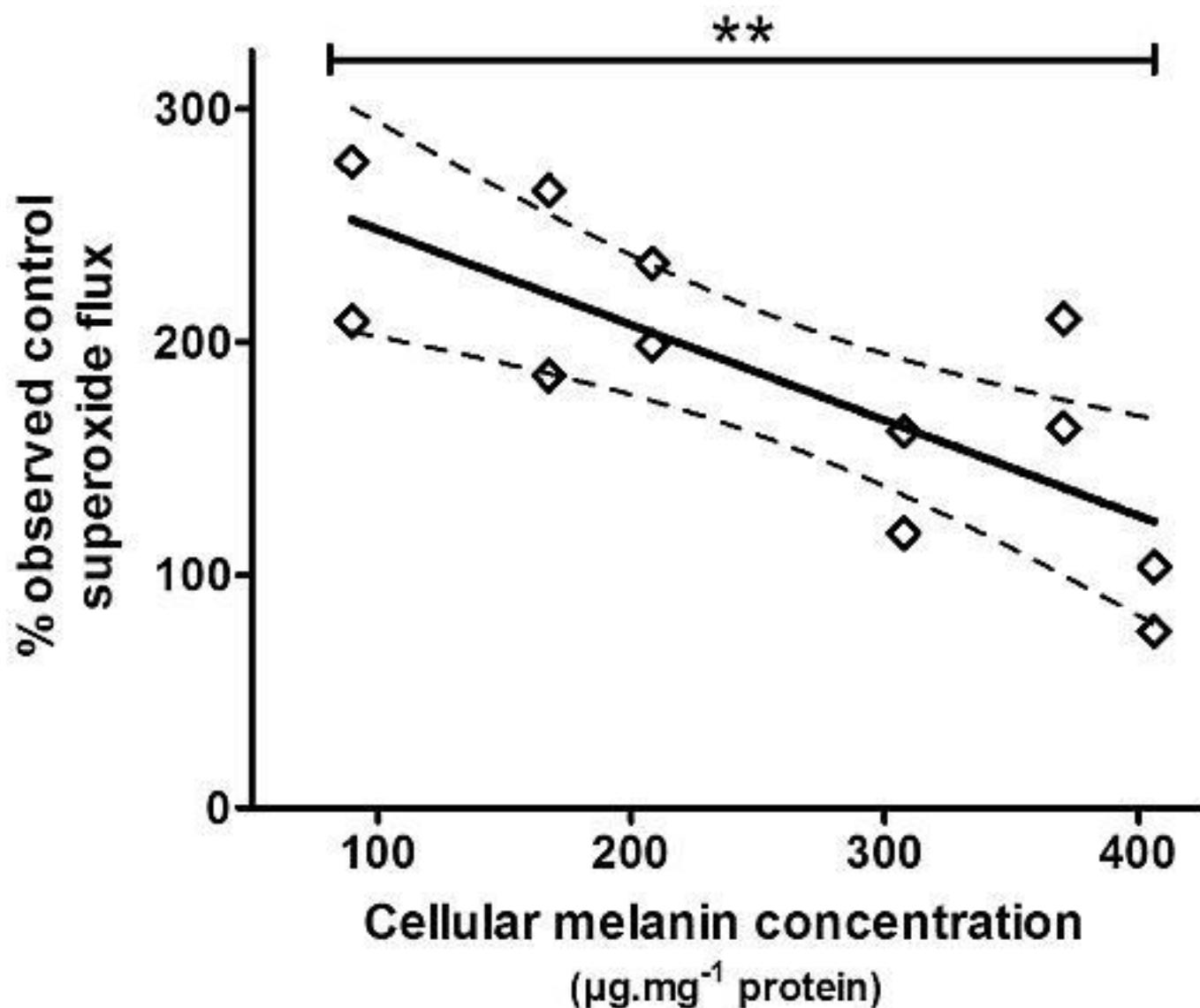


Figure 5: TTFA and 3-NP induced O_2^- flux from pigmented FM55 cells was monitored amperometrically and expressed as a percentage of stimulated O_2^- generated current from non-hyperpigmented FM55 control cells. Normalised flux was plotted against the corresponding sample's observed melanin concentration. Following a single day of pigmentation treatment which resulted in a cellular melanin content of $89\mu\text{g.mg}^{-1}$ protein, an immediate increase in O_2^- flux was observed which gradually diminished simultaneous to increasing cellular melanin concentration. A significant inverse relationship between observed flux and melanin concentration (denoted by **, $p < 0.005$) was suggested by Pearson product-moment correlation analysis (Pearson $r = 0.7730$, $p < 0.005$).